CHARACTERIZATION OF ZEARALENONE AND ITS METABOLITES IN OLDER WOMEN AND THE RELATIONSHIP WITH FOOD INTAKE

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ABSTRACT OF THE THESIS

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The environmental estrogen, zearalenone (ZEA), is found in the food supply from *Fusarium* fungal contamination in grains. Zeranol (Ralgro©), a synthetic form of the metabolite α-ZAL, is an FDA approved growth promoter for use in beef cattle. Animal studies have found ZEA to be associated with reproductive impairment. Circulating concentrations of ZEA and its metabolites [α-zearalenol, zeranol, β-zearalenol, β-zearalanol and zearalanone] have not been previously examined in adults and could have an estrogenic effect. The aim of this research was to characterize the free and total Z metabolite concentrations in human serum and urine samples, and determine associations with food intake. A cross-sectional analysis in 48 women (25-69 years, body mass index of 25 ± 5 kg/m^2) was conducted. An HPLC/MS/MS technique analyzed for metabolites and an average of three 24-hour food diaries was used to assess intakes. Re-analysis of the metabolites was performed using more strict quantitative criteria. The total (free and conjugated) summed metabolite concentration for urine and serum was 43.8 ± 37.8 ng/mL and 1.8 ± 0.9 ng/mL, respectively. The detection of urinary metabolites ranged from 13-88% and 46-100% for free and total metabolites, respectively. In serum,

detection ranged from 17-92% and 17-100% for free and total, respectively. ZEA showed the highest detection rate of all metabolites in both serum and urine. Women with highest (10-17 oz/day) versus lowest (0-4 oz/day) meat intake had higher total serum metabolite concentrations (2.0 ± 0.9ng/mL) compared to those with low intakes (1.1± 0.8 ng/mL) ($p<0.05$). After re-analysis with more strict criteria, concentrations for urinary and serum total metabolites were 27.7 ± 22.2 ng/mL and 1.0 ± 0.7 ng/mL, respectively. The detection rates for urinary metabolites ranged from 2-88% (free) and 8-100% (total). Serum metabolite detection ranged from 2-45%(free) and 4-96%(total). Despite the lower detection rate and concentrations, the same significant relationship with meat intake remained. There is a wide range of ZEA and metabolites present in the serum and urine of adult women and total serum concentrations are associated with meat intake. More investigation into the effects of ZEA exposure in humans is necessary.

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Introduction to Zearalenone

Historical Identification

Zearalenone (ZEA) (Figure 1) was discovered, identified and named by two separate groups of investigators in the 1960s. Christensen et al. found symptoms associated with ZEA in livestock feed more than 50 years ago (1963)[1]. In 1963, herds of young swine in Minnesota that consumed pelleted feeds and were found to have symptoms including: tumefaction of the vulva, prolapsed vagina, and hypertrophy of the mammary glands. The same feed was then fed to guinea pigs and white rats, both of which developed enlarged uteri. In 1964, a herd of swine that was fed grain (30% mold ridden corn and 70% sound corn) developed the same set of symptoms [1]. Christensen and colleagues were able to isolate compounds from the moldy corn, which were not isolated from the sound corn, deemed F-1 and F-2. F-1 was confirmed through various reactions to be ergosterol. F-2 was purified and absorbance spectrum was identified[1].

Urry et al. used nuclear magnetic resonance and mass spectrometric measures to identify the chemical structures of the same compound, F-2, later naming it zearalenone (6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid lactone) due to its structural name in combination with the name of the fungus it is produced by (*Fusarium graminearum* [teleomorph *Gibberella zeae*])(1966)[2].

Chemical Properties

ZEA is an enantiomer of 6-β-resorcyclic acid lactone I, a member of the class of β-resorcyclates. It was identified as a white crystalline substance, with the chemical molecular breakdown of $C_{18}H_{22}O_5$, with a melting point of 164-165°C[2]. It was also insoluble in water, but soluble in aqueous alkali, ether, benzene, and alcohols. Urry and

colleagues also found that five products resulted from the reduction of ZEA[2]. The reduction metabolites include: α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and zearalanone (ZAN) (Figure 1). More recent studies have confirmed these chemical properties, and have also indicated that it is chemically stable. ZEA can withstand 120°C for 4 hours, however is completely reduced in buffer solution at 225°C for less than 30 minutes[3]. Baking, roasting, and fermentation by lactic acid have been shown to decrease concentration levels.

Classification

ZEA can be considered under various classifications, one being a major class of mycotoxins. Bennett and Klich suggest a definition for the umbrella term mycotoxin as "low-molecular-weight natural products produced as secondary metabolites by filamentous fungi", which can be toxic at low concentrations (2007)[4]. Mycotoxins can also be classified in various ways, depending on whether a clinician, cell biologist, chemist, physician is doing the classification, which organ system it can affect, it's chemical structure, and the illnesses it can cause. ZEA can be classified as: a mycotoxin because it is produced by the fungus genus *Fusarium*, a lactone due to its chemical structure, and a mycoestrogen due to its biological activity[4]. The term mycotoxin may be an inaccurate portrayal of the compound because even though it has comparable biological activity to the estrogen, 17-β-estradiol (E_2), it may not be toxic.

Sources

Sources of ZEA include cereal crops, as it is a compound that is biosynthesized through a polyketide pathway by different fungal species under the genus: *Fusarium*. In particular, ZEA is predominantly produced by and is a secondary metabolite of: *Fusarium graminearum*, and *Fusarium culmorum,* which are regular contaminants of

cereal crops worldwide[5]. ZEA can also be biosynthesized by *Gibberella zeae,* an anamorph, of *Fusarium graminearum* [6, 7].

A synthetic form of the metabolite α-ZAL, called zeranol (Ralgro©), has been used as an anabolic agent for both sheep and cattle [8]. This synthetic form was patented in the United States by some of the researchers who originally gave "zearalenone" its name.

Regulations

In 1989, Zeranol, the synthetic form of ZEA used as a growth promoter, was banned by the European Union (EU) [4]. It remains a Food and Drug Administration (FDA) approved growth promoter and there are currently no FDA regulations regarding ZEA in the United States.

Established tolerable daily intake (TDI) for ZEA is 0.5µg/kg-body weight/day [9, 10] whereas its maximum limits in foods in the EU range from 20 to 350 µg/kg (Table 1). The TDI is established by World Health Organization and Food and Agricultural Organization committee that determines the limits on food additives and contaminants utilizing evidence based findings.

Occurrence

Over the last 40-50 years more information has become available about ZEA because of improving and developing methodologies to measure its concentration. High performance liquid chromatography (HPLC), is a technique used to separate components of a mixture or sample, thus being able to identify and quantify them. Another easy, convenient and relatively inexpensive method for analysis is utilizing an enzyme linked immunosorbant assay (ELISA) to measure concentration.

An HPLC analysis of feeds from Oceania and Asia geographical locations for mycotoxins found the most frequently identified mycotoxins to be ZEA and deoxynivalenol (DON)[11]. The samples tested were diverse, ranging from cereals such as corn, wheat, and rice, to processed by-products: soybean meal, corn gluten meal, dried distillers grains and other fodder, including straw, silage and finished feed. The average concentration of ZEA was 148.2 μg/kg. ZEA occurred in 47.5% samples, and of those positive samples the average concentration was 311.6 μg/kg[11]. The maximal ZEA concentration was 16712 μg/kg. This analysis also found the highest positive correlation between DON and ZEA was found in in corn samples. This suggests a wide range of concentrations in a large percentage of the samples examined.

The ELISA methodology was used for the quantitative determination in a study investigating 253 samples including: maize, wheat, barley, silage, pig urine and meat in Croatia [12]. Positive samples were found in maize, wheat and fattening feed. The maximal concentration of ZEA was found in maize samples with an average concentration of 411 ± 860 μg/kg, and a maximum of 5522 μg/kg[12]. Measurable levels were found in male and female pig meat and urine, but differences between genders were not significant. The above study utilized ELISA methodology measured ZEA in the samples but did not quantify its metabolites.

While ZEA is the most common resorcyclic acid lactone (RAL) found in feed, its metabolites have also been detected. These metabolites as well as conjugated metabolites have been found to be measurable in silage, wheat, and barley.

Another study aimed to determine the occurrence of various mycotoxins, trichothecenes and mycoestrogens, and their ‘masked mycotoxins’, or metabolic

byproducts, in cereal based foods to then estimate the exposure to the Belgian population [13]. A total of 174 cereal-based samples were analyzed for mycoestrogen contamination. Categories of cereal based foods included: fiber-enriched bread, bran-enriched bread, breakfast cereals, oatmeal, and popcorn. A sample of 3083 people provided information about their consumption of these foods through a national consumption survey. DON was found in 85% of fiber-enriched breads, 44% bran enriched breads, 58% breakfast cereals, 38% oatmeal samples. DON is a major component of wheat based products, and while it is commonly found in these samples, all were low concentrations levels compared to the EU maximum levels, except in breakfast cereals which had samples that exceeded this value. ZEA was found in lower percentages of the samples, compared to DON, however many more samples exceeded the EU maximum level 50 μg/kg. ZEA occurred in 44% fiber enriched bread samples (29 ± 54 μg/kg, maximum 230 μg/kg), 39% bran enriched bread samples (38 ± 52 μg/kg, maximum 157 μg/kg), 52% breakfast cereal samples (76 ± 165 μg/kg, maximum 450 μg/kg), 58% popcorn (9 ± 19 μg/kg, maximum 51 μg/kg) and 62% oatmeal samples (41±55 μg/kg, maximum 85 μg/kg)[13]. ZEA metabolites were measurable in smaller percentages in these food samples. Deterministic exposure assessment, calculating lower and upper bounds found that the highest exposure was to the mycoestrogens, zearalenone and its metabolites. It was found that high consumers of cereal foods exceeded the established daily TDI. While consumption of the cereal foods likely exceeded the TDI levels and consumption of oatmeal and popcorn were below the TDI, it is likely due to the lower consumption rates for these two foods.

Because ZEA and other mycoestrogens frequently occur in crops, they can then be found in runoff, and transferred to water sources surrounding the agricultural areas. Additionally, if ZEA is ingested and metabolized by humans and animals it can be excreted in the urine and feces, which also have the potential to contaminate water sources. A recent review of surface water contamination found numerous studies that measured the concentration of phytoestrogens and mycotoxins in water sources including: drainage, river, lake and streams in areas in the United States and various countries in Europe [14]. Among the various studies, the levels found in surface waters were relatively low, but were found to be the highest in the summer and autumn seasons, post-harvest. Concentrations of ZEA ranged from 0.3 ng/L to 44 ng/L. The relative potency of the compounds was also investigated and utilizing the relative potency, the investigators then calculated an estradiol equivalent of the compounds. ZEA and metabolites were found to have higher potency compared to the other phytoestrogens investigated. α-ZAL was found to be the most potent metabolite with a mean of 4.75 x 10^{-1}, and β-ZAL was the least with a mean of 2.25 x 10^{-3}, where E_2 equals 1. The calculated estradiol equivalents of zearalenone ranged from 0.01-0.76 ng/L. Although the concentrations of zearalenone and its metabolites were found in low ranges in the surface waters, their relative potencies were higher than other compounds investigated.

Metabolism

There are two phases, first identified in 1978, of ZEA metabolism: phase I, the reduction of ZEA (Figure 2), and phase II, the conjugation of ZEA and the products of phase I metabolism to gluconurides (Figure 3). The investigation of ZEA metabolism in rat liver model and found that conjugation of metabolites, or phase II, to be the main route of metabolism[15].

Various other investigators later confirmed the two phases of metabolism in other species. ZEA metabolism in species include: rat, rabbit, beagle, monkey and human was compared, and it was found that rabbit and man predominately excrete ZEA and conjugated metabolites in the urine, while rat, beagle and monkey predominately excrete ZEA in bile[16]. During this research, it was also found that β-ZAL, ZAN, and conjugated glucuronides are the major metabolites. The route of excretion was confirmed when Fitzpatrick and colleagues dosed rats with ZEA, and found 55% of the administered dose in feces, while 15-25% was excreted in the urine (1988)[17]. ZEA is mostly excreted in the free form [17]. Bories et al. examined glucuronidation and sulfation of ZEA and metabolites in rat and pig livers and found that both glucurono- and sulfonoconjugates are produced, and that conjugation occurs through the aromatic ring (1991)[18].

Phase I metabolism involves hydroxylation of the ketone group of ZEA which results in the reduced alcohol, either α-ZEL or β-ZEL[19]. These reduced alcohols can be further metabolized to the ZAN. Phase I of metabolism, occurs in the liver and includes the reduction of zearalenone to aromatic metabolites in humans, and aliphatic in rats [20, 21]. Hepatic reduction occurs through a dehydrogenase, either 3α-HSD or 3β-HSD [22].

Phase II metabolism involves glucuronidation and sulfation of either zearalenone or the metabolites formed in phase I[19]. Phase II includes conjugation of the metabolites with glucuronic acid or sulfate and can occur in both liver and intestine [22].

A recent review of ZEA exposure and intoxication of farm animals, specifically looking into toxicokinetics, toxicity of RALs, and consequences, summarizes metabolism

of ZEA in various species [22]. Metabolism from oral consumption of ZEA occurs at different levels: pre-absorptive, absorptive and post-absorptive and the pre-absorptive level of metabolism differs between species depending on whether the animal is mono-gastric and poly-gastric[22]. The absorptive and post-absorptive levels of zearalenone metabolism generally occur in the intestinal mucosa and liver. There were numerous studies that investigated various species, including rats, poultry, pigs, and cattle, with measureable amounts of dietary ZEA and measured free and conjugated metabolites in different matrix samples including urine, blood plasma, bile, and milk. In all species and all sample matrix types, higher percentages of metabolites are conjugated, either with glucuronic acid or sulfate. The authors suggest that while the methods for analyzing and detecting this compound and its metabolites are changing and improving over the last 30 years, the metabolite pattern remains comparable, and α-ZEL and β-ZEL appear to be the main reductive metabolites of zearalenone while the others are of minor importance[22]. The mean proportion of ZEA and its metabolites, including free and conjugated, was calculated in various species in different matrix samples. Predominantly, most samples, regardless of species or sample type, were found to have ZEA, α-ZEL and β-ZEL, and the other metabolites did not occur or were at very low levels. [22]

Olsen et al. found 100% ZEA in both blood plasma and urine of gilts recovered as glucuronic acid conjugates of ZEA and α-ZEL(1985) [23]. Prelusky et al. had similar findings in plasma and milk of cows, but found ZEA, α-ZEL and β-ZEL, and while 100% was conjugated, it was not specified what compounds were conjugated to (1990)[24]. Winkler et al. published works in 2014 and 2015 consistent with the finding that

conjugated metabolites predominate, but extended the analysis to all ZEA and its metabolites[25, 26]. Kleinova et al. found similar results in urine of heifers (2002)[27].

Danicke et al. examined the effects of intravenous (IV) bolus administration of 10 mg ZEA/kg body weight of piglets over 14 days (2005)[28]. The investigators observed the entero-hepatic cycling process and found maximal plasma concentration after 2.73 hours of administration, and noticed that ZEA and α-ZEL are highly recycled[28]. They found fecal excretion to increase greatly at 48 hours post administration and continue after 72 hours. After 2 weeks of administration, ZEA and α-ZEL were below the limit of detection in liver, bile, and urine.

Estrogenic Activity

ZEA and its metabolites can be classified as mycoestrogens or environmental estrogens because their structures resemble that of the estrogen, E_2 (Figure 4). Of even more importance, ZEA and its metabolites have also been found to have similar biological activity compared to E_2. A suggested ranking of the estrogenic potential of the metabolites is as follows: α-ZAL > α-ZEL > β-ZAL >ZEA > β-ZEL [19].

A method to determine the relative estrogenic activity of ZEA compared to E_2, is a recombinant yeast estrogen screen (rYES), an in vitro assay [29]. The rYES was transfected with human estrogen receptor α (ERα) to assess the ability of ZEA to bind to and activate the estrogen receptor. In this assay, both ZEA and E_2 produced sigmoidal curves that had comparable maximal receptor activation, however for E_2 this occurred at 2 μg/L compared to ZEA's concentration of 500 μg/L[29]. This indicated moderate estrogenic activity of ZEA, a half maximal effective concentration ratio of 1: 250 compared to E_2.

In contrast to the majority of studies analyzing how ZEA and its major metabolites interact with estrogen receptors, Molina-Molina and colleagues investigated primarily how they interact with other nuclear receptors, specifically androgen receptors (2014)[30]. Additionally, they examined the estrogenic effects in vitro utilizing MCF7 cells, a human breast cancer cell line. They found that like the natural estrogen E_2, all 6 ZEA compounds stimulated cell proliferation, showing dose dependency [30]. Both α-ZAL and α-ZEL had highest potency of the metabolites, only 3 and 7 fold less than[30] E_2. When the metabolites were examined in relationship to the human androgen receptors, α-ZAL was found to have the most antagonistic effect, and exhibited a full dose response curve in the PALM cell, a bioluminescent line used to examine androgen and anti-androgen effects[30]. This study demonstrates the endocrine disrupting attributes of zearalenone and its metabolites.

The estrogenic activity of ZEA has been compared to that of α-ZAL, by determining their relative binding affinities for human estrogen receptors: ERα and estrogen receptor β (ERβ)[31]. By using the estrogen receptor competitive binding assays, it was found that both compounds had a strong binding affinity for ERα and ERβ, but α-ZAL had an 11 fold greater affinity for ERα and 4 fold greater affinity for ERβ when compared to ZEA. ZEA had a slightly higher affinity for ERβ compared to ERα, and α-ZAL showed an opposite preference for binding to ERα. Molecular modeling utilizing the protein databank examined binding conformations. ZEA and α-ZAL occupy the active site of the estrogen receptors as E_2[31]. Their phenolic ring occupies same space as A ring of E_2; α-ZAL would be more potent estrogenic ligand due to its hydrogen bonding activity.

Assessment of Exposure in Vivo

Acute and sub-acute toxicity of ZEA has been examined in rats, mice, and piglet models. Duca et al. found that Sprague Dawley rats dosed with 25 mg/kg body weight of ZEA intra-peritoneally (IP) had altered mRNA expression involved with detoxification of xenobiotics (2012)[32]. Ouanes et al. found that IP doses ranging from 2 to 4 mg/kg body weight in BALB/C mice for 24, 48, and 72 hours resulted in chromosome abnormalities of bone marrow cells, which occurred through nuclear translocation after estrogen receptor binding (2005)[33]. Pistol et al. conducted a feeding trial in piglets, administering feed contaminated with 250 mg/kg feed ZEA for 18 days (2014)[34]. They found that this diet containing ZEA significantly reduced levels of pro- and anti-inflammatory markers at both the gene and protein level, resulting in overall hepatic immunosuppression[34]. Nikaido et al. alternatively researched subcutaneous injection of 0.5-10 μg/kg/day ZEA into pregnant mice (2004)[35]. Of note, ZEA caused accelerated onset of puberty, and prolonged estrus cycle, along with prolong effects on the reproductive tract and mammary glands[35]. Acute exposure can result in altered gene expression, altered inflammatory processes and can impact puberty.

Short term ZEA exposure to zebrafish, ranging from 100 ng/L to 3200 ng/L for 21 days has been found to impair reproduction [29]. Although this exposure had no effect on body weight or length and there were no gonadal differences in the zebrafish, it induced a concentration dependent decrease in spawning frequency, and fecundity, and an increase in plasma vitellogenin (VTG). VTG is a gene for an egg oocyte protein precursor that is usually muted in male fish, but can be induced with estrogen exposure, and is used as a marker of endocrine disrupters[36]. This demonstrated no severe acute toxic effects of ZEA in vivo.

In another study, Bakos et al. examined ZEA in zebrafish at different ages (2013)[37]. They found that a full life cycle exposure of 0.32-1.00 μg/L resulted in a shifted sex ratio toward female, as well as an increased weight and length of the female fish. A dose of 1ug/L induced VTG, however exposure had no effect on fertility, hatch, survival of gonad morphology. When embryos were exposed to 0.1 μg/L for 5 days, induction of VTG mRNA was observed, but it was found to be significant at 5 μg/L[37]. Exposure of the embryos to much higher concentrations 50-750 μg/L resulted in reduced pigmentation, edema, and body curvature[37]. This suggests more adverse effects at higher concentrations when exposure for a full life cycle.

Fathead minnow embryos with 7-day exposure to 0.002-0.05 μg/L of ZEA had increased body size and edema [38]. This concentration of ZEA up-regulated the genes for growth hormone, insulin-like growth factor, luteinizing hormone, and VTG. The induction of VTG by ZEA was compared to the induction caused by E_2 [38]. Acute exposure in this species produced hormonal effects.

ZEA has also been found to have effects on hematology and immune markers. One study examined the effects of dietary exposure of ZEA on carp[39]. With three experimental dosing groups to juvenile carp for four weeks of feeding with two recovery weeks, no effect was found on growth (weight and length), however hematological parameters were altered. Medium (621 μg/kg) and high (797 μg/kg) doses resulted in significantly lower monocytes and higher granulocytes [39]. Granulocytes and monocytes are important immune effector cells, indicating a possible immune system effect of ZEA. Medium doses of ZEA resulted in significantly lower hemoglobin after two recovery weeks.

In another animal model, Tiemann et al. examined pigs fed contaminated feed (4-358 μg/kg feed) for 35 days and observed inhibition of the proliferation rate of splenocytes and elevated hemosiderin without any clinical symptoms (2006)[40]. Alm et al. also investigated pigs and contaminated feed, at lower levels of contamination (0.1-6.3 μg/kg feed) for the same duration, but examined specifically the oocyte maturation (2006)[41]. They found oocyte alteration leading to reproductive failure at this exposure concentration. Doll et al. performed a feeding experiment and examined piglets consuming contaminated feed for 42 days (1.2 mg/kg feed)(2005)[42]. An increased in uterus weight as well as alteration in serum parameters was observed. Chronic exposure can affect the spleen functionality as well as reproduction.

ZEA has also been found to be a possible teratogen, or a compound with the ability to alter the developing embryo and fetus. ATP binding cassette (ABC) transporter genes have been shown to be modulated by ZEA. ABC transporter proteins are broadly expressed and some members of this protein family are involved with the transport of nutrients, drugs, and xenobiotics across membranes. Koraichi and colleagues utilized a pregnant rat model to study the transcriptional modulation of ABC transporters by ZEA (2012)[43]. Daily exposure to ZEA through subcutaneous injection of 1mg/kg/day for 15 days affected the maternal fetus mRNA and protein expression of various ABC transporters. It also inhibited fetal liver expression of the proteins, suggested that fetus development could be impacted by ZEA exposure.

Assessment of Exposure in Humans

While a variety of species and ZEA exposure have been examined, studies in humans are rare. A recently published review investigated in vivo studies of *Fusarium*

mycotoxins over the last decade [44], and found only 1% of studies investigated effects in humans.

A feasibility study examining the relationship between urinary mycoestrogens, breast development and menarche found measurable levels of ZEA and its associated metabolites in urine samples of 163 New Jersey girls, ages 9-10 years [45]. There was 78% detection of urinary free mycoestrogens, with ZEA having the highest level and a 55% detection rate, while zeranol had a 20% detection rate and lower levels. Levels were similar between seasons of recruitment. Six girls reported popcorn intake prior to the study day and had significantly higher levels of ZEA and total metabolites. Beef intake was also found to be associated with higher urinary ZEA. Overall, mycoestrogens were detectable in a large proportion of participants and these findings should be further addressed in larger studies with less homogenous sample and in longitudinal designs.

A case study involving a healthy 27-year-old male examined the effect of an 8-day diet on urinary DON and ZEA [46]. The special diet consisted of a 2-day baseline which was cereal restricted, 4 days of high cereal intervention followed by 2 days of cereal restriction, 24-hour urine samples were collected daily, and compounds were measured using LC-MS/MS methodology. Enzymatic hydrolysis of the urine samples allowed for the analysis of free ZEA and Zearalenone-glucuronides (ZEA-GlcA). Of interest, the foods consumed in the high cereal diet, naturally containing mycotoxins were: breakfast cereals, wheat bran, maize porridge, maize flour, bread, wheat beer and popcorn, with a summed intake of 10 μg ZEA/day, resulting in an intake of 0.2 μg/kg body weight below the TDI[46]. The majority of the ZEA was consumed as the maize porridge (94%) during the lunch meal. The average total ZEA in urine during the

interventions diet was 0.39 μg/L with a range of 0.30-0.59 μg/L[46]. Compared to the baseline cereal restricted diet when the concentration of urinary total ZEA was 0 μg/L during the high cereal diet the concentration of ZEA increased significantly due to diet. Zearalenone-14-glucuronide (ZEA-14-GlcA) was directly determined in spot urine samples 3-10 hours after lunch and was never found in the first morning samples[46]. This case study suggests rapid formation and excretion of the conjugate metabolites.

Another study to assess human exposure of numerous mycotoxins associated with food consumption based on direct measurement of urinary biomarkers was conducted in a Belgian population [47]. This included assessment of 239 adults and 155 children and analyzed morning urine samples. There was no detection of ZEA, its metabolites or its conjugated metabolites in the urine samples of children. α-ZEL was detected in only one sample (1/239, 5.0 ng/mL, corrected for Cr-4.3 ng/mg), and β-zearalenol-14-gluconuride (β-ZEL14GlcA)(2/239, mean 0.8mg/mL, 0.9ng/mg, range 0.-1.0 ng/mL) in two samples of Belgian population[47]. ZEA, β-ZEL , and conjugated forms (SEN14GlcA, α-ZEL7GlcA, α- ZEL14GlcA) were not detectable in urine samples[47]. Dietary exposure to the mycotoxins DON and ochratoxin A was estimated from the urinary concentrations, and then compared to the established TDI levels. Due to the lack of detection of urinary ZEA, the dietary exposure was unable to be calculated. This study suggests that ZEA may not be detectable in all samples, despite consumption of naturally occurring mycotoxins in food.

A review investigating mycotoxin exposure and its effects on infant and young child growth specifically in Africa found no studies examining ZEA in this population[48]. The author suggested this may be due to the lack of biomarker

availability, unlike other mycotoxins, alfatoxin and fumonisin, that are found to be associated with alterations in infant and young child growth impairment. Nevertheless, the authors of this review found no epidemiological studies looking at zearalenone and maternal, infant and young child exposure or growth impairment.

Rationale and Hypothesis

Since its discovery and identification in the 1960s, the compound ZEA and its metabolites has been investigated extensively in food, livestock, and in experimental models. It is understood that ZEA occurs through natural fungal contamination of grains. Grains and feeds containing this compound can be introduced into the food supply. The past 50 years have allowed for development of new methods to detect and analyze this compound in various sample matrices. Despite the methodological advances, the research conducted in humans is lacking.

As emphasized in a recent review, only 1% of *in vivo* studies specifically investigating *Fusarium* mycotoxins (the source of ZEA) have been performed in humans [44]. Another short review examining dietary mycotoxins and their co-occurrence suggests that ZEA is a commonly occurring and significant mycotoxin[49]. ZEA and other mycotoxins have complex interactions that have not been adequately studied and these interacting and ubiquitous mycotoxins could negatively impact human health, posing a carcinogenic risk, and reproductive concerns.

From a public health standpoint, ZEA is a mycoestrogen of concern due to its estrogenic activity. Despite the low concentrations of mycotoxin in foods (μg/kg), there can be adverse health outcomes and the chronic effects of long-term exposure are not fully understood. Lee et al. calls for the need of a system process known as risk assessment to characterize the potential adverse effects of ZEA and mycotoxins in general to best assess regulations regarding consumption (2015)[50].

With development of new methodologies comes the ability to detect and quantify previously poorly identified and undetected substances. Most commonly found in the

literature to determine ZEA concentration in plasma and urine are HPLC methods used in combination with tandem mass spectrometric (MS/MS), ultraviolet, and fluorescence detection[51]. Some methodologies allow for detection of ZEA and its major metabolites, while others are only able to detect ZEA. The development of a sensitive and specific method for quantitative determination the concentrations of ZEA (liquid chromatography MS/MS and ultra-high performance liquid chromatography-high resolution-mass spectrometry (U)HPLC-HR-MS) and its metabolites in serum and urine, was found to be inexpensive, with easier sample preparation, compared to the methods previously used[51]. This method development study utilizes a technique similar to the methodology used in the present investigation (HPLC-MS-MS); however this is the first description detailing the present methodology. This suggests a specific and sensitive new methodology used to determine accurate concentrations of ZEA, specifically in serum and urine.

The literature on ZEA exposure indicates potential risk for detrimental health outcomes and the lack of adequate assessment in humans demonstrates a major gap in the current knowledge and need for further investigation. The main objective of this thesis research was to characterize ZEA and its metabolites in the urine and serum of adults.

This thesis addresses the following specific aims regarding ZEA and its metabolites:

1. To characterize serum and urinary ZEA and its metabolites in women, and establish the ratio of free to conjugated metabolites.
2. To determine whether ZEA is influenced by dietary intake of specific food groups.

It was hypothesized that ZEA and metabolites will have measurable levels in serum and urine in an adult population, and would be influenced by dietary intake.

Methods

Subjects and Study Design

A retrospective cross-sectional analysis was conducted in healthy, pre- and post-menopausal women. Subjects were previously recruited at Rutgers University through local newspaper, electronic and radio station advertisements for clinical studies involving weight loss and bone health. Anthropometrics (height, weight, BMI) were measured by trained individuals on a balance scale and stadiometer in the clinical laboratory at Rutgers University. Serum and urine samples were used from the Osteoporosis Weight Loss and Endocrine (OWLE; NIH-AG12161) data set, aliquoted and frozen for storage at -70°C. Certified phlebotomists performed the blood draws. Usual dietary intake was defined as the average of three 24-hour dietary recalls, which were also conducted by trained individuals. Dietary recalls were conducted on the day of urine and serum sample collection.

The protocols were approved by the Institutional Review Board of Rutgers University (New Brunswick, NJ) and all subjects provided written informed consent prior to any study procedure.

Biomarker Analysis

Samples were transferred to the Chemical Analysis Facility of the Occupational and Environmental Health Sciences Institute (EOHSI) for analysis. Urinary and Serum

mycoestrogen analyses were conducted. These data was analyzed for ZEA and other conjugates that eluted in the same region, and also for single peaks (utilizing stricter quantitative analysis).

Briefly, ZEA and its metabolites were measured using an HPLC-MS-MS technique. 0.25 ml sodium acetate buffer (pH=4.65) and 10µl of β-glucuronidase from *Helix* pomatia was added into 1ml of urine or serum. The enzymatic deconjugation was conducted in a water bath at 37 °C overnight. Then liquid-liquid extraction was performed on a Visiprep™ DL SPE vacuum manifold to isolate analytes from urine or serum. Chem Elute 1ml cartridge was used in the liquid-liquid extraction. The injection volume is 20µl. A Thermo LTQ mass spectrometer was interfaced to a Finnigan Surveyor Autosampler plus and Finnigan Surveyor MS Pump plus for separation and quantitation of zeranol and its metabolites. The analytes were quantified using an LC/MS/MS method. The precursor ions are: m/z 321(α-ZAL and β-ZAL), m/z 319 (α-ZEL, β-ZEL and ZAN) and m/z 317 (ZON) and the quantitation ions were m/z 277 and m/z 303 (α-ZAL and β-ZAL), m/z 275 and m/z 301(α-ZEL, β-ZEL and ZAN), m/z 273 and m/z 299 (ZEA). Atmospheric negative pressure chemical ionization source was used to ionize ZEA and its metabolites before introduction into the mass spectrometer.

Urinary metabolite concentrations, also measured at the EOHSI lab, were corrected for dilution by specific gravity. When concentrations were below the limit of detection, zero values were used in the data analysis. The sum of metabolites was calculated as the sum of ZEA, α-ZAL, β-ZAL, α-ZEL, β-ZEL, and ZAN.

Analysis of Livestock Feed and Selected Food Samples

An ELISA analysis of ZEA content in selected livestock feed, common grains for human consumption, milk, and meat samples was assessed by ELISA (Ridascreen

Zearalenon R1401, R-BioPharm, Darmstadt, Germany). All reagents, including standards, for the enzyme immunoassay were in the test kit. A microtiter plate spectrophotometer was utilized for quantification. Livestock feed samples, including heifer and pig (gestational sow, grower, creep, lactation) feeds, were obtained from Animal Care Program on the GH Cook Campus of Rutgers University (New Brunswick, New Jersey). Meat samples, including ground beef, and grass fed ground beef, burger patty meat, 98% ground turkey, and rib eye steak, were obtained from local grocery stores. R-BioPharm's protocol procedures for sample preparation were followed. Results from ELISA analysis are shown in Appendix I.

Statistical Analysis

Statistical analyses were conducted using the SAS statistical package (SAS Institute, Cary, NC, USA; v 9.3). All values are reported as mean ± SD. Descriptive statistics was used to characterize the data. Differences between groups were examined with one-way ANOVA. One-way ANCOVA was used to control for covariates such as age. When the F ratio was significant, Tukey's post-hoc analysis was performed. Significance was considered at a p value < 0.05.

Results

The analysis included 48 female subjects and their demographics are shown in Table 2. Subjects ranged in age from 25-69 years old, they had an average body weight of 65.5 ± 15.6 kg, and average BMI of 25 ± 5 kg/m^2.

Serum concentrations of ZEA and its metabolites (Z metabolites) are shown in Table 3. Both the free concentrations as well as the total concentrations are reported as ng/mL. The total concentration equals the concentration of free combined with the concentration of conjugated metabolites for each given metabolite. The summed metabolite concentration indicated for either free or total, equals the addition of ZEA, α-ZAL, β-ZAL, α-ZEL, β-ZEL, and ZAN.

In the serum, free metabolites were detectable in 96%, and total metabolites were detectable in 100% samples, when considering the summed total metabolite concentrations. Free detection rates of the six individual compounds ranged from 17-92%, with free ZEA having the highest detection rate. Detection rates of the total concentration of the six individual compounds ranged from 16.6-100%, again with ZEA being detected at the highest rate. The sum of free Z metabolite concentration in serum was 0.7 ± 0.5 ng/mL. The sum of the total (free+conjugated) Z metabolite concentration in serum was 1.8 ± 0.9 ng/mL. Despite overall low concentrations of ZEA and metabolites in the serum, there was a high detection rate across the compounds.

The urinary concentrations of ZEA and related compounds are presented in Table 4. The values reported are corrected for specific gravity. Similar to serum measurements, both free and total values are reported. Detection rates of free urinary metabolites ranged from 13%- 88% with ZEA having the highest free concentration detection rate of any

individual metabolite in the urine. Detection rates of the total (free+conjugated) urinary Z metabolites range from 46-100%, with ZEA detected in 100% of samples. The sum of free metabolite concentration was 0.9 ± 0.9 ng/mL. However, the concentration of total urinary metabolites was much higher; the total summed metabolite concentration was 43.8 ± 37.6 ng/mL. The maximum level of summed total urinary concentration was 192.8 ng/mL, which was 36 times higher than the maximum concentration measured in serum.

Women were categorized by BMI status: 4% were underweight (BMI < 18.5 kg/m^2), 60% had normal BMI ranging between 18.5-24.9 kg/m^2, 23% had a BMI ranging from 25.0-29.9 kg/m^2 and were categorized as overweight, while 13% were obese with a BMI of 30 kg/m^2 or greater. Grouping BMI categories into Normal Weight (BMI <25 kg/m^2) and Overweight-Obese (BMI ≥ 25 kg/m^2) groups a trend was found ($p=0.076$) for total serum concentration of metabolites between BMI groups (Figure 5A). In addition, when age was used as a covariate the difference between groups remained a trend ($p<0.1$). Those with Normal Weight had higher total metabolite concentration 1.4 ± 0.8 ng/mL) compared to the Overweight-Obese (0.9 ± 0.8 ng/mL). However, free serum concentration level did not differ between BMI groups (Figure 5B).

When comparing Normal Weight to Overweight-Obese women, the normal weight women were found to have higher total ZEA serum concentration and lower free ZEA serum concentration ($p<0.03$). Total α-ZAL serum concentration is also higher in normal weight women ($p<0.05$).

Dietary intake through the compilation of 24-hour dietary recalls was also analyzed in these women. When subjects were categorized by meat intake into Low (0-4 ounces/day), Medium (5-9 ounces/day) and High (10-17 ounces/day), serum summed Z

metabolites was significantly different between High and Low meat intake groups ($p<0.05$) (Figure 6). High meat intake groups had higher total serum concentrations (2.03 ± 0.08 ng/mL) compared to low meat intake groups (1.09 ± 0.08 ng/mL). No differences were found when subjects were analyzed by other grain or other types of food intake.

Reanalysis with More Strict Chromatographic Elution Criteria

In an effort to ensure that only the Z metabolites are reported (and not other similar compounds eluted in the same region of the chromatogram), the data was reanalyzed two years later utilizing stricter criteria in of the peak elution time. To be potentially more conservative, the elution time was limited as a precaution. The stricter criteria greatly reduced the detection rate and concentration levels of ZEA and associated compounds in serum and urine. This method included assays for free and total detection, with a higher detection limit of the total concentration compared to the detection limit of the free assay. This resulted in instances where a free concentration (high sensitivity assay) was detected, but no total concentration was found (lower sensitivity). In these instances, when the total concentration is greater than or equal to the free concentration, the free concentration value was reported for the total concentration.

Measurements of the concentrations with strict criteria are shown in Tables 5 and 6. The detection levels of free Z metabolite concentration in serum ranged from 2-45%, however ZEA remained the highest detected free metabolite. Total metabolite detection ranged from 4-96%, and ZEA, again, was the highest. The analysis of free α-ZAL only resulted in one sample with detectable levels. The summed concentration of free Z metabolites in the serum was 0.6 ± 0.4 ng/mL, while total was 1.0 ± 0.7 ng/mL. The

maximum level detected in serum with strict criteria was 3.5 ng/mL compared to 5.3 ng/mL in the original analysis.

Analyses of the urine samples had similar reductions in detection rate and concentration levels. The detection range for free urinary metabolites ranged from 2-88% and 8-100% rates were found with the total concentrations. ZEA had the highest detection rate for both free and total in urine. The summed free and total urinary metabolite concentrations were 0.6 ± 0.9 ng/mL and 27.7 ± 22.2 ng/mL, respectively. The maximal concentration detection in urine was 83.3 ng/mL compared to the original analysis 192.8 ng/mL.

With the re-analysis of the samples, similar results were found when data was analyzed by BMI groups Normal Weight (BMI <25 kg/m^2) and Overweight-Obese (BMI ≥ 25 kg/m^2) , with higher total serum concentrations(1.2 ± 0.7 ng/mL) in Normal Weight compared to Overweight-Obese (0.7 ± 0.6 ng/mL) (Data Not Shown). In addition, the new analysis yielded similar findings when subjects were grouped by meat intake (Figure 7). Higher serum summed total metabolites (1.2 ± 0.8 ng/mL) were found with high meat intake, 10-17 ounces/day, when compared to low meat intake (0-4 ounces/day) (0.6 ± 0.6ng/mL) ($p<0.05$).

Discussion

Zearalenone (ZEA) and its metabolites (Z metabolites) (α-ZEL, β-ZEL, α-ZAL, β-ZAL, ZAN) are found in cereal grains through natural fungal contamination, and α-ZAL is used as a growth promoter in beef cattle in the United States. These compounds can be considered mycoestrogens due to their similar structure in comparison to E_2, and α-ZAL has been found to have the highest estrogen potential.

To the best of our knowledge, this study is a unique investigation of ZEA and metabolites measured in human serum and urine samples, utilizing a highly sensitive and specific methodology. We found a large percentage of samples with detectable levels of ZEA and its metabolites in urine and serum samples of healthy women, independent of the criteria used for the analysis. We were able to characterize detection rates of the metabolites and calculate summed concentrations to determine circulating and excreted levels of the estrogenic compounds. We were also able to characterize both free and conjugated forms of the compounds.

It was consistently found in both serum and urine that ZEA had the highest detection rate obtained from the HLPC/MS/MS methodology. The other metabolites (α-ZEL, β-ZEL, α-ZAL, β-ZAL, ZAN) were detectable in the samples, but at lower rates compared to ZEA. This is likely because ZEA is the parent compound that can be reduced to other metabolites, or conjugated with glucuronic acid or even sulfates.

The first analysis of the samples resulted in high concentrations in serum, but more so in urine, with high maximum levels: 5.3 ng/mL and 192.8 ng/mL, for serum and urine respectively. It is thought that this analysis may have included compounds other than ZEA and its metabolites. During this analysis, conjugates that eluted in the same

region as ZEA and its metabolites may have been included, resulting in higher detection rates and concentrations levels. However the secondary analysis with stricter elution criteria narrowed the range for peaks, minimizing or eliminating the conjugates that could have eluted in the same region. With the more strict criteria, we can be confident that the values reported only reflect ZEA and its metabolites.

There is a difference in concentrations of ZEA and metabolites when analyzed by BMI category. With an increase in BMI, there was an increase in the serum free concentration of metabolites. This occurred despite a trend for a decline in total metabolites. These results remained when the data was reanalyzed with more strict criteria. This suggests that ZEA and metabolites have similar relationships with weight and BMI as E_2 does.

It is also known that females with a BMI classified as obese and overweight have higher levels of serum E_2, compared to those with lean BMI [52]. Overweight and obese women have higher serum E_2 compared to women with a normal BMI, possibly due to higher aromatase enzyme activity [53]. To date the relationship between weight status and BMI and ZEA in humans has not yet been established. More research and further studies into the relationship with weight status and BMI are needed.

We also found a significant difference between serum ZEA concentrations and meat intake. Participants with low meat intake, categorized as 0-3 servings per day, had significantly lower concentrations of summed serum metabolites when compared to those with high meat intakes, 7-10 servings/day. When these data was re-analyzed using the strict criteria, the finding remained significant. This is important because despite lower detection rates and concentration levels of metabolites, the relationship with dietary meat

intake remains the same. This is similar to the finding from the Jersey Girls study**,** which analyzed young healthy girls and found beef to be a predictor of ZEA [45]. However that relationship was found from urinary ZEA analysis. The present investigation, suggests that the use of serum biomarkers of ZEA and metabolites may also useful in determining exposure or risk assessment.

A separate and secondary analysis had lower detection rates and concentration levels, shown in Appendix II. It was thought that this occurred because of possible metabolite breakdown due to a freeze/thaw cycle (thawed ~24 hours) that occurred during power outages during a hurricane (Hurricane Sandy 2012). In addition, the detection rate was very low in this dataset. It is suggested that ZEA compounds breakdown over time and if not kept frozen.

Bandera and colleagues investigated a larger population of young girls (n=163) and reported on urinary mycoestrogens and the free form of the metabolites (2011)[45]. These data were analyzed in our laboratory using the original criteria (not strict). In this study of girls, there was a 78% detection rate for the summed free urinary metabolites with an average level of 1.3 ± 3.7 ng/mL and a range of 0.03-29.8 ng/mL with ZEA having the highest detection (55%). In comparison, in our adult population we were able to detect ZEA and metabolites in more samples, and at higher levels. In our original analysis using the same criteria as the girls, the women had an 89% detection rate for summed free urinary metabolites with an average concentration of 0.9 ± 0.9 ng/mL and ranged 0.01-9.9 ng/mL. In our adult population, urinary concentration levels of summed free metabolites were detected in a larger percentage of the population, compared to the study conducted in young girls [45]. Methodologies used in our study and in this

previously conducted study were similar and the same lab conducted sample biomarker analysis. Compared to this earlier study, a limitation of our study was the smaller sample size.

A case study was performed in a single healthy young adult male[46] with an unique dietary intervention of high cereal diet and also included analysis of other mycotoxins. Interestingly, this study found an increased urinary ZEA concentration due to an interventional diet. LC-MS/MS methodology was utilized in this analysis. At baseline there was no detectable urinary ZEA, and after the naturally ZEA containing diet, an average urinary ZEA concentration of 0.39 μg/L (range over 7 days of daily measurements: 0.30-0.59 μg/L) was found. These investigators analyzed total urinary concentration ZEA and conjugated forms, but there were no reports on serum measurements[46]. Our results compared to this case study were 112 and 71 times higher for our original and more strict criteria analysis, respectively, with larger range despite the observational nature of our study.

In contrast to our study and the studies previously mentioned, an assessment of exposure to mycotoxins in the Belgian population using LC-MS/MS validated methodology found no detection of ZEA or metabolites in children [47]. In the adult Belgian population only α-ZEL and β-ZEL-14-gluconuride were detectable, however only in 1 and 2 samples, respectively. It is difficult to draw conclusions from such low detection rates.

The established provisional maximal TDI for zearalenone is 0.5μg/kg body weight per day. The 48 women from this analysis had an average weight of 65.5 kg, thus their maximal TDI of ZEA would be 32.75 μg ZEA/day. Consumption of more than this

TDI could result in negative effects, as indicated by numerous studies in animals. Some studies have even found that exposure at concentrations lower than the TDI have adverse effects

Two instances of rodent studies with exposure to ZEA through intra-peritoneal injection at levels 25 mg/kg body weight and 2-4 mg/kg body weight, found altered mRNA and chromosomal alteration, respectively [32, 33]. Both of these amounts are lower than the TDI recommended: 0.5 μg/kg body weight/day (500 mg/kg body weight /day), and still have adverse effects. An investigation of pregnant mice that were subcutaneously (SC) injected with 0.5-1.0 mg/kg resulted in an accelerated onset of puberty, with a prolonged estrus cycle and accelerated mammary gland differentiation in the offspring[35]. These doses of ZEA were also below the TDI. However, it is important to note that these studies used IP and SC injection, which is not subject to the metabolism and degradation that a metabolite introduced through dietary exposure goes through.

Dietary exposure in animals has been investigated, but most are in excess of the TDI established for humans and have adverse effects. A feeding trial with piglets that either consumed a diet contaminated with 250 parts per billion (PPB) of ZEA or a control diet, found decreased pro- and anti-inflammatory markers (TNF-α, IL-8, IL-6, IL-1β, IL-10 and IL-4) with the contaminated feed[34]. The decrease in immune response was found with a diet in great excess of the human TDI. Moreover, the feed was measured to contain 316 PPB or μg/kg ZEA, meaning an additional ZEA content that was already in the diet prior to adding 250 PPB of ZEA for treating the animals. Similarly, a study of dietary exposure to ZEA in carp found that a diet in excess of the TDI (621 and 797 μg/kg) had an adverse effect, with altered granulocytes and monocytes[39]. Dietary

exposure, also in excess of the TDI, ranging 4-358 μg/kg ZEA in feed altered spleen function of pigs[40]. Even a slight excess of the TDI in the diet (0.1-6.3 μg/kg), had negative impacts on reproduction in pigs [41].

Investigations in dietary exposure less than the TDI also indicate adverse effects. Piglets consuming a feed with 1.2 mg/kg ZEA had increased uterine weight and altered serum parameters [42]. Taken together, the dietary exposure studies in animals generally examining amounts in excess of the TDI for humans are associated with negative health effects. However, even with amounts below the TDI negative effects were reported. This indicates the need for further investigations in humans that can help lead to better regulations regarding ZEA contents of foods. If concentrations below the TDI result in adverse effects, and there are currently no guidelines for regulations of foods in US, the US population could unknowingly be exposed to ZEA and metabolites, causing negative impacts on health.

There are limitations of the present research. This study was a retrospective, cross sectional analysis and therefore it cannot establish causality. We did not examine any health effects in this sample due to its small size. A strength of the data is that whether or not the data is analyzed with possible other compounds or related ZEA conjugates or there is stricter criteria, both analysis showed similar trends with BMI and to be higher with greater meat intake.

The lack of current knowledge on the assessment of ZEA and metabolites in humans requires further investigation. This investigation found high concentrations of ZEA and metabolites in serum and urine. Future research should include interventional

studies, with ZEA containing diets and populations with a wide age and BMI range, and examine a broad array of health outcomes.

Particularly important, studies should be conducted in population at risk of health problems upon ZEA exposure such as pregnant women, men and young children and infants. Pregnant women consuming compounds could negatively impact embryonic and fetal development. In men, testicular function could be negatively affected; it has been shown that can ZEA inhibit testosterone biosynthesis[54]. In all adults, an endocrine disruptor, like ZEA, could negatively impact reproduction and act as a carcinogen. Infants and young children are of concern due to their ability to consume a greater intake per kg body weight compared to adults, and intake and impact growth and development. Overall, more information on mycoestrogens exposure is necessary in order to develop regulatory and safety measures that will protect the health of individuals.

Table 1: European regulations for zearalenone in food [9]

Unprocessed corn	350 µg/kg
Corn flour	200 µg/kg
Unprocessed cereals other than corn; Corn intended for direct human consumption, corn-based snacks and breakfast cereals	100 µg/kg
All product derived from unprocessed cereals intended for direct consumption (excluding processed corn-based foods)	50 µg/kg
Cereal based baby foods (including processed corn-based foods) for infants and young children	20 µg/kg

Note: there are no regulations of zearalenone in the USA

Table 2: Participant demographics

Characteristic	*n* (%)
Gender	
Female	48 (100)
Age (years)	
25-44	7 (15)
45-64	38 (79)
64+	3 (6)
Menopausal Status	
Pre	19 (40)
Post	29 (60)
BMI (kg/m^2)	
< 18.5 (Underweight)	2 (4)
18.5-24.9 (Normal)	29 (60)
25-29.9 (Overweight)	11 (23)
> 30 (Obese)	6 (13)

Table 3: Serum concentrations (ng/mL) of zearalenone and metabolites in adult women (n=48)

Metabolite	Free					Total (Free+Conjugated)				
	% Detection	Mean	SD	Min	Max	% Detection	Mean	SD	Min	Max
ZEA	91.6	0.351	0.213	0.114	0.988	100	0.722	0.431	0.157	1.720
α-ZAL	47.2	0.369	0.175	0.137	0.745	72.9	0.437	0.263	0.120	0.960
β-ZAL	8.3	0.211	0.063	0.148	0.270	16.6	0.434	0.308	0.162	0.960
α-ZEL	2.0	0.457	0.286	0.238	0.780	62.5	0.509	0.352	0.170	1.163
β-ZEL	35.4	0.402	0.199	0.176	0.774	39.6	0.380	0.326	0.136	0.946
ZAN	31.2	0.518	0.315	0.147	0.968	91.6	0.332	0.246	0.114	0.968
SUM	95.8	0.725	0.465	0.148	1.770	100	1.796	0.891	0.458	5.300

Table 4: Urinary concentrations(ng/mL) of zearalenone and metabolites in adult women (n=48)

Metabolite	Free					Total (Free+Conjugated)				
	% Detection	Mean	SD	Min	Max	% Detection	Mean	SD	Min	Max
ZEA	87.5	0.582	0.882	0.028	3.971	100	24.435	20.848	1.123	77.410
α-ZAL	27.1	0.109	0.047	0.033	0.184	75	4.395	3.505	0.488	13.615
β-ZAL	12.5	0.081	0.047	0.027	0.152	62.5	10.396	20.677	0.691	95.730
α-ZEL	16.6	0.122	0.040	0.069	0.193	54.2	1.852	3.324	0.141	16.927
β-ZEL	20.8	0.608	1.417	0.040	4.620	45.8	4.241	3.636	0.123	13.752
ZAN	39.5	0.225	0.295	0.0004	1.221	97.9	6.311	5.1122	0.189	21.500
SUM	89.6	0.868	0.868	0.011	9.898	100	43.749	37.566	1.851	192.794

Metabolite levels corrected for specific gravity

Table 5: Serum concentrations (ng/mL) of zearalenone and metabolites in adult women (n=48) with strict criteria applied

Metabolite	Free					Total (Free+Conjugated)				
	% Detection	Mean	SD	Min	Max	% Detection	Mean	SD	Min	Max
ZEA	44.9	0.345	0.201	0.148	0.98749	95.9	0.644	0.422	0.148	1.720
α-ZAL	2.0	0.254		0.254	0.254	4.1	0.449	0.276	0.254	0.644
β-ZAL	4.1	0.265	0.007	0.260	0.270	4.1	0.265	0.007	0.260	0.270
α-ZEL	8.2	0.614	0.336	0.238	0.990	16.3	0.688	0.483	0.236	1.613
β-ZEL	20.4	0.428	0.225	0.190	0.831	22.4	0.407	0.224	0.190	0.831
ZAN	18.4	0.529	0.309	0.156	0.968	32.7	0.477	0.2955	0.126	0.968
SUM	67.3	0.602	0.430	0.148	1.491	98.0	1.015	0.676	0.148	3.543

*Total Concentration ≥ Free Concentration

Table 6: Urinary concentrations (ng/mL) of zearalenone and metabolites in adult women (n=48) with strict criteria applied

Metabolite	Free					Total (Free+Conjugated)				
	% Detection	Mean	SD	Min	Max	% Detection	Mean	SD	Min	Max
ZEA	88.2	0.582	0.880	0.028	3.944	100.0	24.436	20.867	1.123	77.412
α-ZAL	2.0	0.152		0.152	0.152	7.8	6.921	3.991	0.937	9.068
β-ZAL	7.8	0.095	0.049	0.034	0.152	19.6	5.320	5.891	0.105	19.185
α-ZEL	7.8	0.126	0.025	0.094	0.154	29.4	1.878	2.515	0.123	9.167
β-ZEL	11.8	0.198	0.198	0.041	0.522	31.4	3.561	4.187	0.041	13.419
ZAN	9.8	0.055	0.055	0.011	0.140	11.8	0.128	0.1095	0.011	0.280
SUM	90.2	0.624	0.880	0.011	3.944	100.0	27.706	22.158	1.327	83.301

*Total Concentration ≥ Free Concentration. Metabolite levels corrected for specific gravity

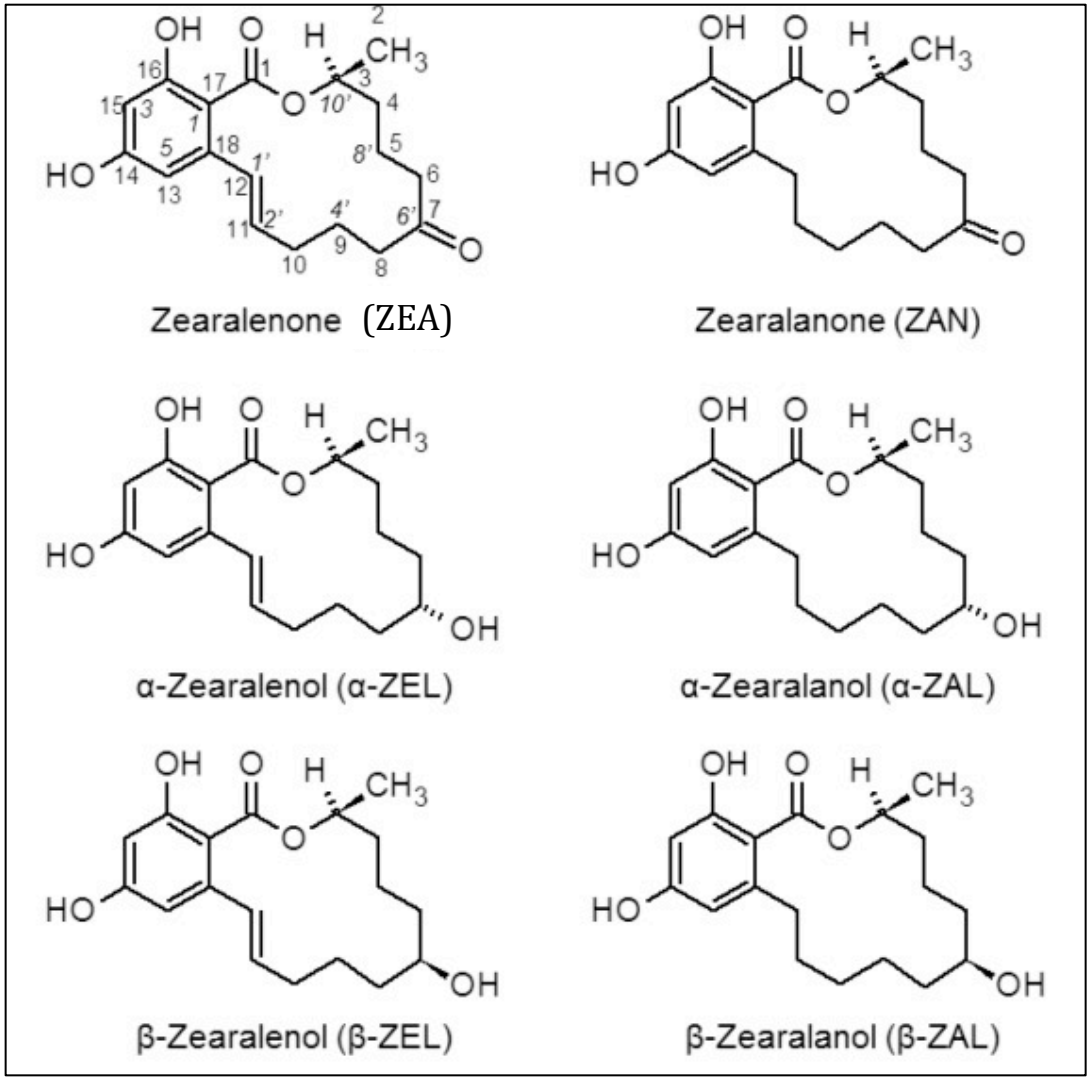

Figure 1: Structure of Zearalenone (ZEA) and its metabolites [22]. Reprinted from Food and Chemical Toxicology, Vol 84, Dänicke, S &J. Winkler, Diagnosis of zearalenone (ZEN) exposure of farm animals and transfer of its residues into edible tissues (carry over), 225-249, Copyright (2015), with permission from Elsevier.

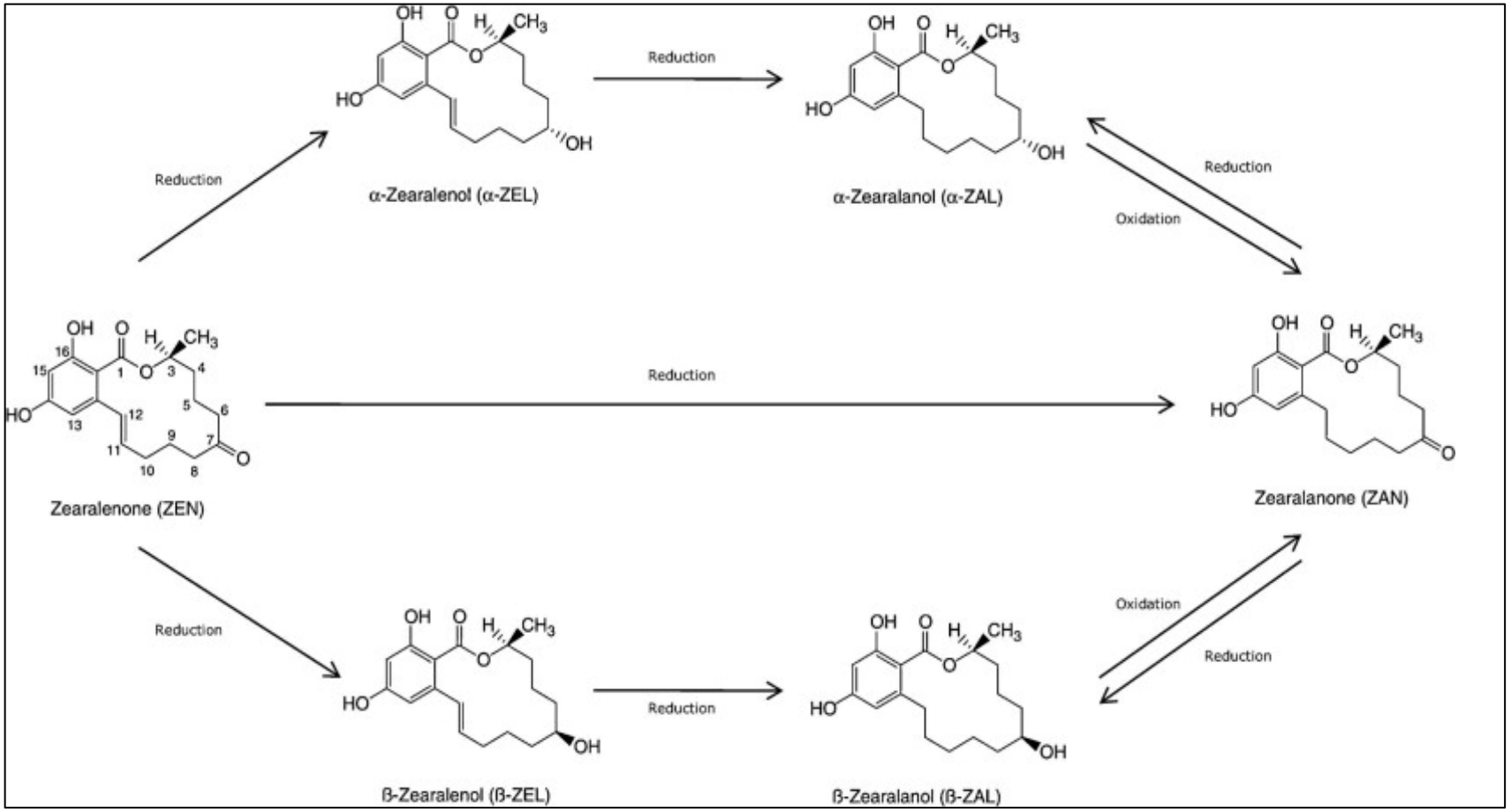

Figure 2: Metabolism of ZEA Phase I: Reduction [30]. "Reprinted from Food and Chemical Toxicology, Vol 74, Molina-Molina et al., Assessment of estrogenic and anti-androgenic activities of the mycotoxin zearalenone and its metabolites using in vitro receptor-specific bioassays, 233-239, Copyright (2014), with permission from Elsevier."

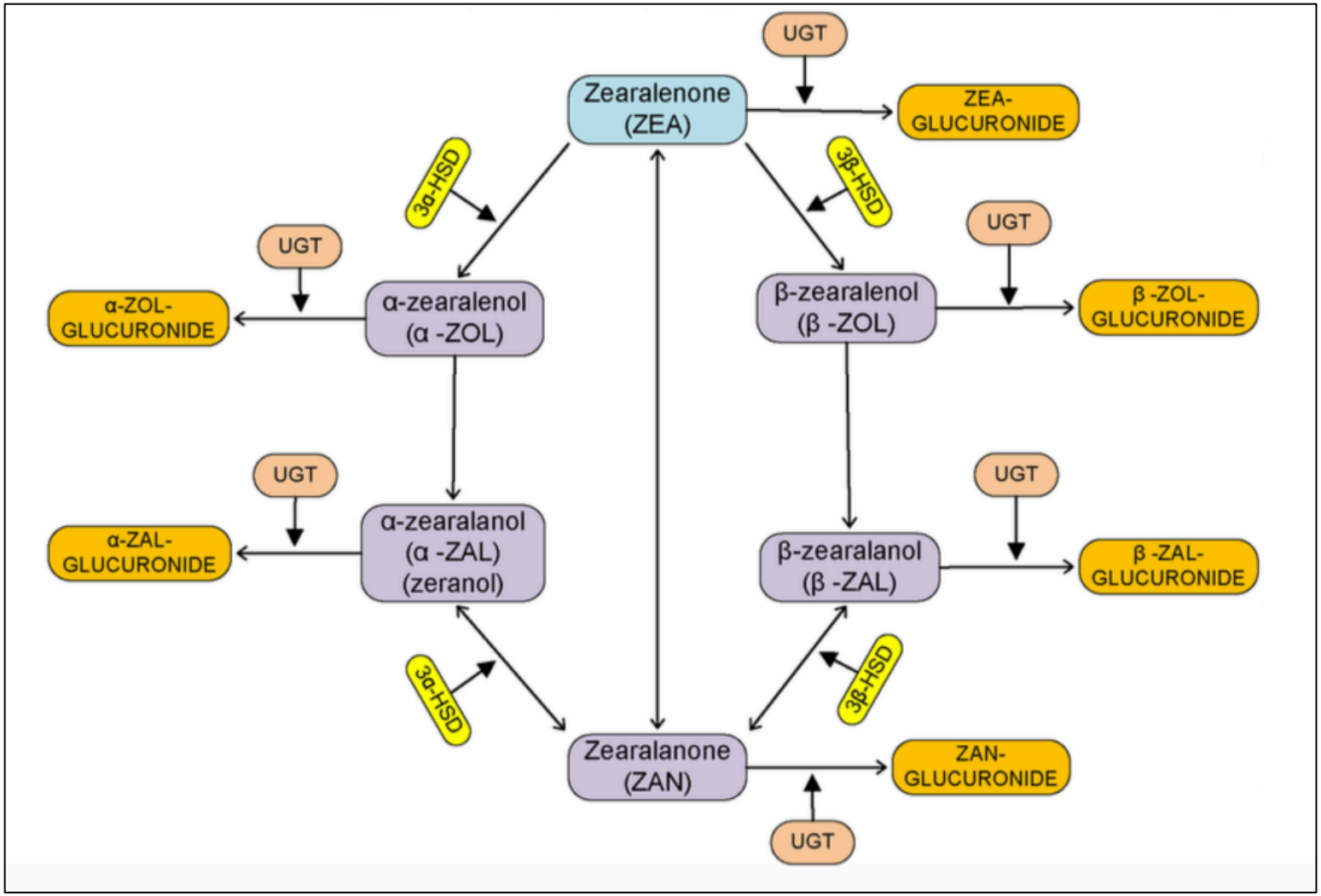

Figure 3. Metabolism: Phase II-Conjugation [19]. Muhkerhee et al. 2014 Original publisher: PLOSONE Image reproduced in accordance with open-access policy of PLOSONE

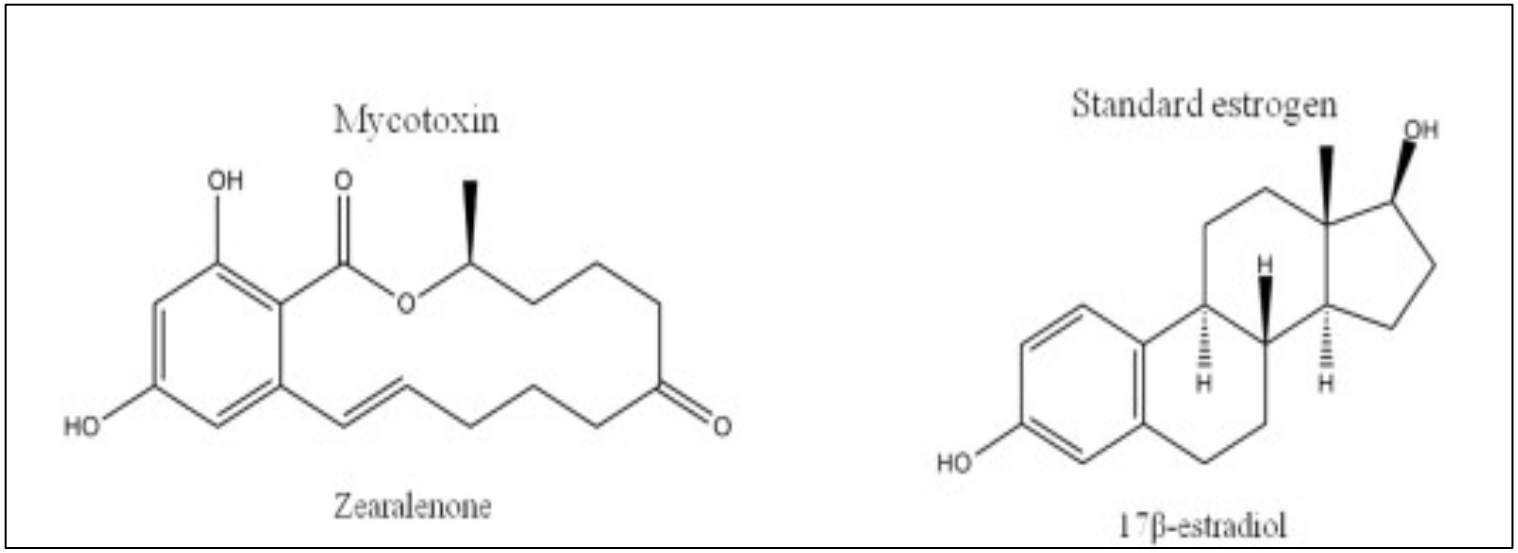

Figure 4. Structure of ZEA Compared to Structure of 17 β-Estradiol [20]. "Reprinted from Environment International, Vol 81, Jarošová, b. Et al., Phytoestrogens and mycoestrogens in surface waters — Their sources, occurrence, and potential contribution to estrogenic activity, 26-44, Copyright 2015, with permission from Elsevier."

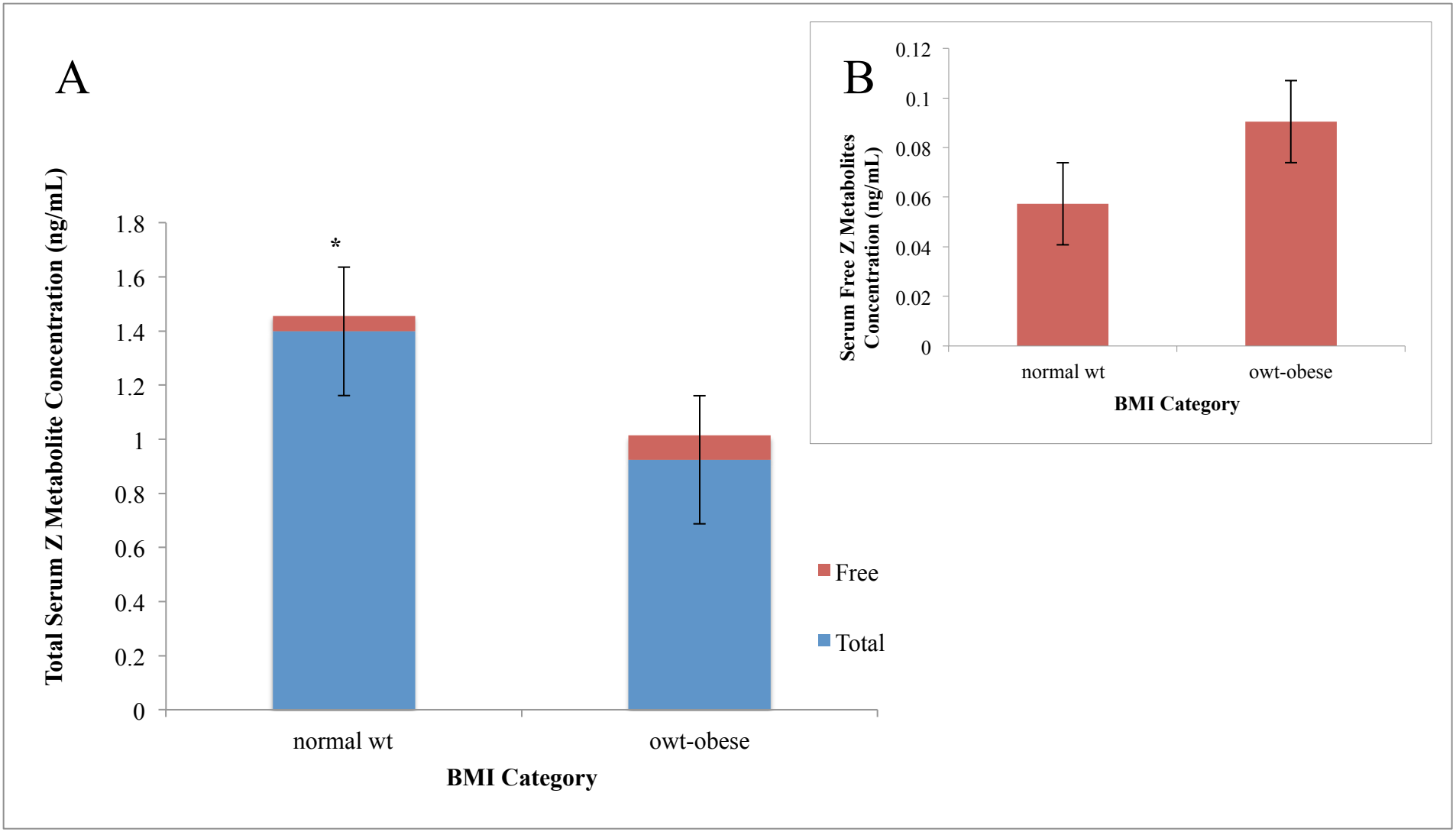

Figure 5. Sum of serum Z metabolites grouped by BMI categories[1]

BMI categories: normal weight (wt) (BMI < 25; n=31) or overweight (owt) and obese (BMI ≥ 25; n=17).

A. Total serum metabolite concentration * Trend for differences between groups before ($p=0.076$) and after ($p<0.1$) correcting for age.

B. Serum free metabolite concentration

[1] Not significant when values with stricter criteria elution are used in this analysis

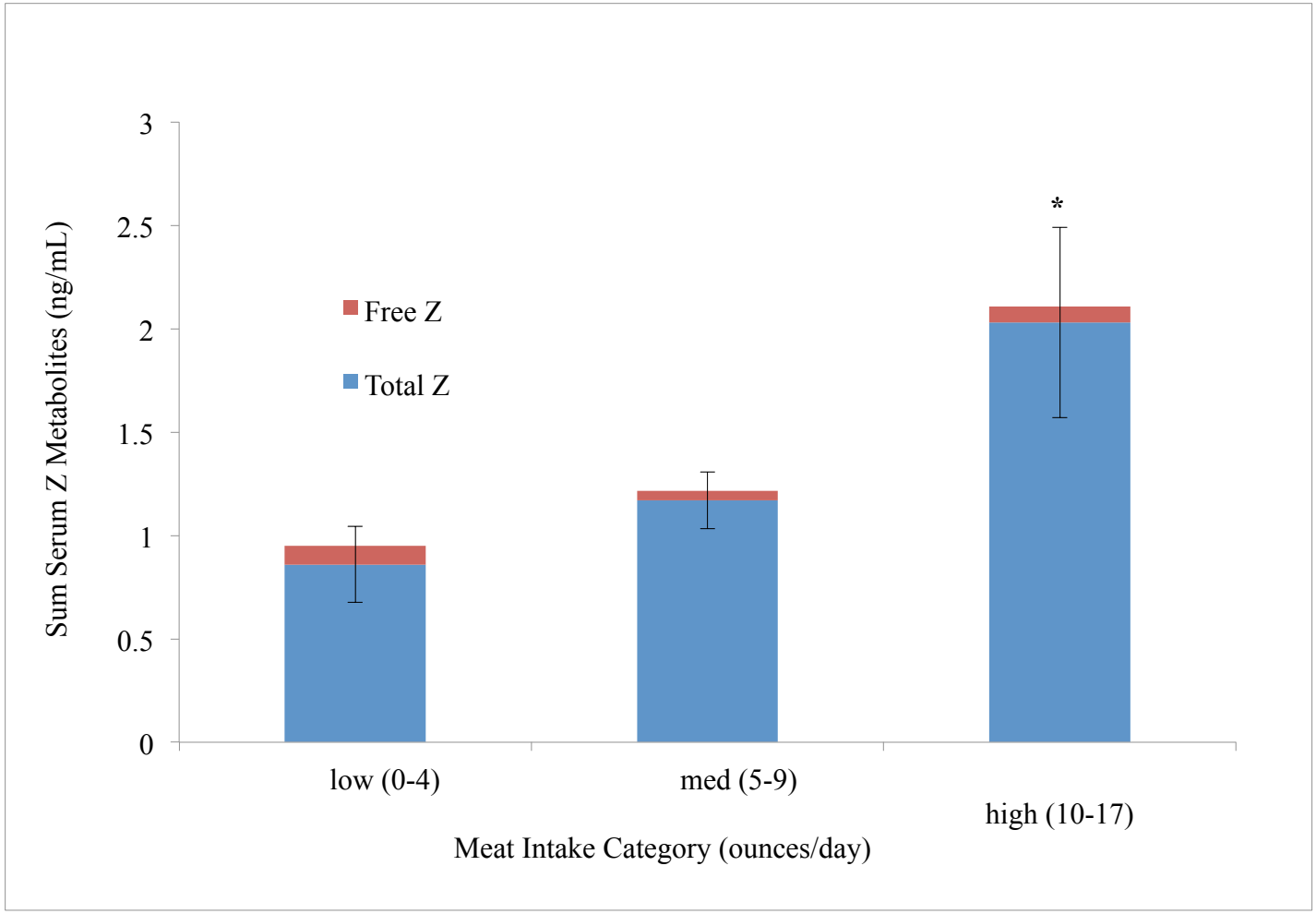

Figure 6. Sum of serum Z metabolites grouped by meat intake.[1,2]

[1]Subjects categorized by low (n=22), medium (n=19), and high (n=7) meat intake.

[2]BMI did not differ between groups (low 25 ± 6 kg/m^2; medium 24 ± 4 kg/m^2; high 27 ± 8 kg/m^2).

*Differs from low intake ($p < 0.05$).

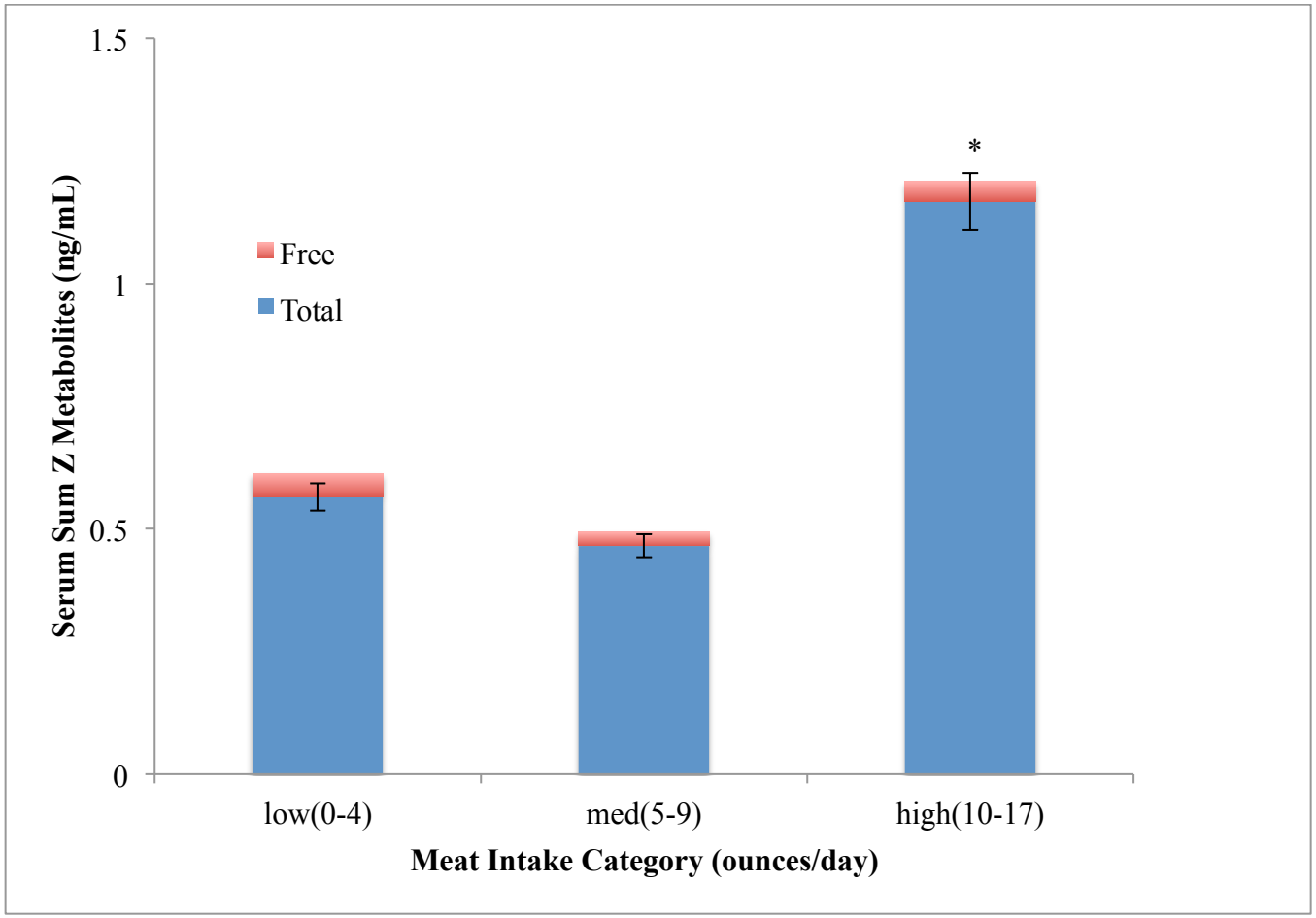

Figure 7. Sum of serum Z metabolites grouped by meat intake- strict criteria.[1,2]

[1]Subjects categorized by low (n=22), medium (n=19), and high (n=7) meat intake.

[2]BMI did not differ between groups (low 25 ± 6 kg/m^2; medium 24 ± 4 kg/m^2; high 27 ± 8 kg/m^2).

*Differs from low intake ($p < 0.05$).

Appendix I: ZEA analysis of selected grains and meats

Objective: To determine the concentration of ZEA found in livestock feed and meat samples.

Methods: The quantitative analysis of ZEA in livestock feed and meat samples was performed utilizing competitive enzyme immunoassay (RidaScreen Zearalenon, R1401, R-Biopharm, Germany). Livestock feed samples, including heifer and pig (gestational sow, grower, creep, lactation) feeds (n=5), were obtained from Animal Care Program on the GH Cook Campus of Rutgers University (New Brunswick, New Jersey). Meat, milk, and grain samples were all obtained from local grocery stores. Meat samples included: ground beef, and grass fed ground beef, burger patty meat, 98% ground turkey, and rib eye steak (n=5). Grain samples (n=13) included: bulgur, couscous, wheat berries, oats, rye berries, brown flax, millet, buckwheat, farro, rice, cornstarch, cornmeal, and infant cereal. Milk samples (n=5) included: cow's milk - fat free, low fat, reduced fat and whole milk; and goat's milk. R-BioPharm's protocol procedures for sample preparation that were specific for different food items were followed.

Results and Conclusions: The ZEA concentrations of livestock feed (n=5) and meat (n=5) are shown in Figures A and B below. The selected milk and grain samples had no detectable concentrations of ZEA (not shown). Our findings of detectable ZEA in local samples of meat and livestock feed provides support for our findings of an association between meat intake and circulating blood concentrations. This should serve as a basis for future studies to determine both the ZEA content in other samples in the food supply and the environment, and to design studies to determine health related outcomes.

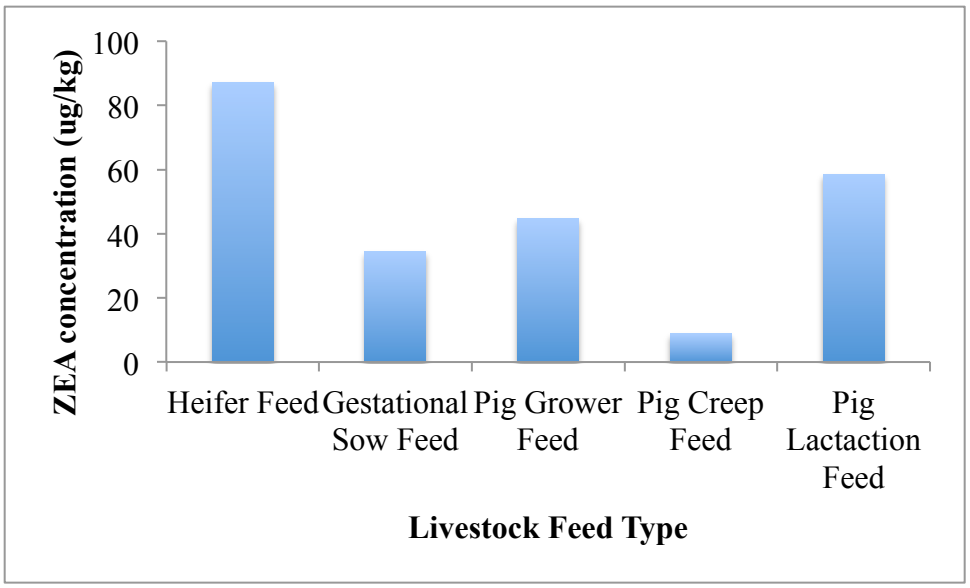

Figure A. ZEA Content of Livestock Feeds. Heifer feed was the only positive feed sample, with concentration above detection limit for feeds.

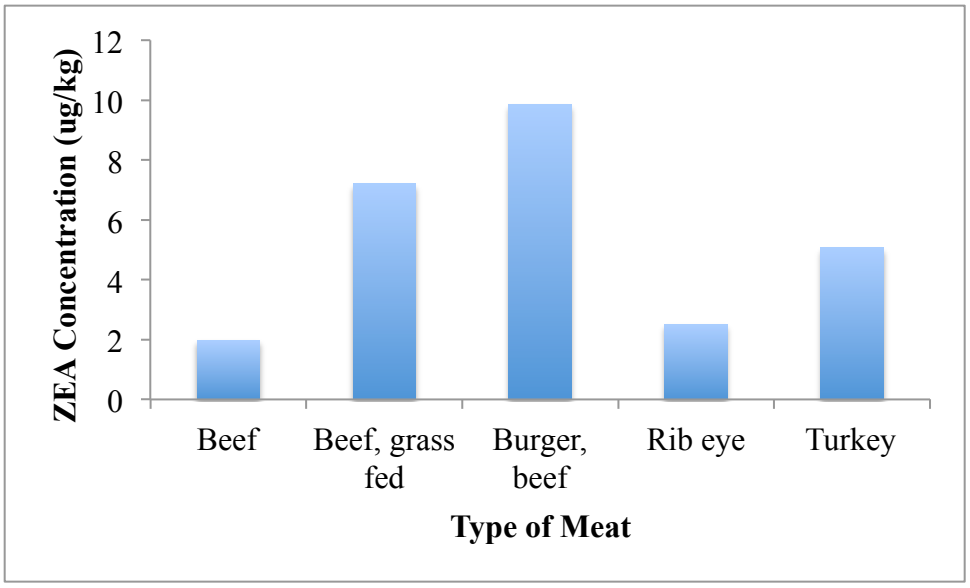

Figure B: ZEA Content of Meat Samples. All reported meat values are above the detection limit.

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